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A FACULTATIVE SPORE-FORMING LACTOSE-FERMENTING ORGANISM FROM IOWA SURFACE WATERS, (B. MACERANS)[1]

By Jack J. Hinman, Jr.,[2] and Max Levine[3]

The occasional presence of sporing lactose-fermenters in water capable of growing aerobically has been reported by Meyer, Ewing, Ellms, Perry and Monfort, and Hall and Ellefson, but very little is known as to the source or biology of these forms.

In the course of routine water analyses at the Iowa State Water Laboratory it appeared that, with chlorinated surface waters the proportion of unconfirmed presumptive tests was excessive, when using preliminary enrichment in lactose broth, and eosin methylene blue agar for confirmation. With litmus lactose agar, however, atypical colonies were not infrequently obtained, which often formed gas when fished to lactose broth and which would therefore be regarded as members of the colon group, on the basis of the Treasury Department Standard. These organisms were invariably negative for gas formation in lactose bile. It occurred to us that possibly aerobic sporing lactose bacilli might be responsible, for a part at least, of these atypical reactions, and attempts were made to isolate them.

Table 1 has been prepared to show the frequency with which presumptive tests upon filtered, chlorinated Iowa city waters were found to be organisms which could not be confirmed as B. coli or B. aerogenes. It appears that those waters which were taken from the Iowa River, or from the Mississippi River below the junction of the Iowa with the Mississippi, are particularly likely to contain these non-confirmed organisms. This apparent peculiarity may be due, however, to the greater number of samples examined from the cities of Burlington, Iowa City and Keokuk where such water is handled.

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[1] Presented before the Iowa Section meeting, November 1, 1921.

[2] Associate Professor of Sanitation, State University of Iowa.

[3] Department of Bacteriology and Pathology, Iowa State College.

From two sources, Iowa City and Burlington, Iowa, 14 pure cultures have been isolated and studied as to their morphological, cultural and other characteristics. Considerable difficulty was encountered in effecting separation from non-fermenting, sporing aerobes, which seemed to grow associatively. The method which proved most successful was (1) to grow in lactose (Andrade) broth, (2) to plate on lactose (Andrade) agar as soon as acid developed, (3) fish acid colonies to lactose (Andrade) agar slants (inoculate sur-

TABLE 1

Fermentation tests on treated Iowa city waters, March 3, 1914, to December 30, 1920

CITY	1 CC. WATER				10 CC. WATER			
	B. coli	B. aerogenes	Positive presumptive tests not confirming	No gas formation	B. coli	B. aerogenes	Positive presumptive tests not confirming	No gas formation
Burlington	22	2	41	651	71	22	584	1852
Cedar Rapids	0	1	7	11	3	4	21	23
Centerville	9	1	14	54	33	5	40	37
Chariton	2	0	2	11	12	1	12	12
Council Bluffs	0	0	2	4	0	0	8	8
Creston	3	2	5	27	11	11	12	34
Davenport	2	0	2	25	12	0	28	80
Fairfield	1	0	5	8	1	0	2	1
Ft. Madison	3	1	2	11	14	7	10	17
Iowa City	35	2	312	3868	62	6	492	1147
Keokuk	11	0	22	101	56	8	258	135
Lenox	3	1	2	2	4	8	2	0
Oskaloosa	0	0	1	7	0	0	5	7
Ottumwa	0	0	4	8	0	0	5	10
Storm Lake	3	0	0	16	18	3	2	21
	94	10	421	4804	297	75	1481	3384

Total number of fermentation tubes	10,566
Total number positive	2,378
Total number positive, non-confirming	1,902
Per cent positive tubes non-confirming	80 per cent
Per cent all tubes non-confirming positive	18 per cent
Burlington, Iowa City, Keokuk:	
Per cent positive tubes non-confirming	85.3 per cent
Per cent all tubes non-confirming positive	17 per cent

face and butt) and incubate the latter for 72 hours observing daily. If pure, a transparent growth with acid on slant and acid and gas in butt will be observed. If non-fermenting spore-formers are present the surface growth becomes opaque. Microscopic examination of Gram's stain of 48 to 72 hour culture on lactose (Andrade) agar should show *no* spores. If spores were present, then the purification process outlined above was repeated, for it was observed that whenever this was the case, a *non-fermenting* aerobic spore former could be isolated while the pure fermenting type did not show spores on this medium in the designated time. The strains isolated resemble B. macerans described by Schardinger.

MORPHOLOGY

Vegetative cells. The organism varies in size on different culture media.

On nutrient agar, the vegetative cells appear (after 24 hours at 37°C.) as rods about as wide as B. coli, but 2 to 4 times as long. The size of the majority being 0.6 by 2.5 μ. They are grouped singly or in pairs; parallel forms were frequently observed and occasionally V forms were noted. The ends are rounded and the bacillus is slightly fusiform.

On lactose (Andrade) agar and in litmus milk the cells are somewhat longer, usually 3 to 4 μ. Spores were not seen.

In lactose (Andrade) broth they appeared especially elongated often measuring 6 and occasionally 8 μ. All of the 14 cultures showed an occasional spore on nutrient agar after 24 hours and after 48 hours at 37°, sporangia and spores were numerous. The endospores are elliptical, their diameter greater than that of the vegetative cells and located subterminally. The size of the majority of spores was 0.8 by 1.4 μ.

STAINING REACTIONS

After repeated observations and comparison with known cultures the organisms were considered to be Gram negative. This was particularly true if the cultures were grown on lactose media. The gentian violet stain is removed with difficulty and the Gram stain may easily be confused. The technique employed was to stain for 1½ minutes with aniline oil gentian violet then with Gram's iodine, to decolorize for 5 minutes with fresh 95 per cent alcohol and to counter-stain with dilute saffranin.

The Gram stain showed the vegetative cells to exhibit a tendency to granulation but in cultures from agar and Loeffler's blood serum no granules were discernible when stained with carbol fuchsin, Loeffler's methylene blue or Albert's diphtheria stain. The vegetative cells stain readily with all of the stains mentioned.

MOTILITY

All of the 14 cultures were found to be motile when examined in a hanging drop from a 16 hour, 37°C. peptone water culture.

SPORE FORMATION

It has already been mentioned that spores were formed readily on nutrient agar in 2 days at the body temperature; it is significant to note, however, that spores could not be demonstrated in lactose agar or milk even after long incubation.

Three cultures were inoculated into lactose broth, lactose agar and plain agar and incubated at body temperature for 48 hours. No spores were visible in the lactose broth or lactose agar but they were numerous on plain agar.

On another occasion six cultures were inoculated into two batches of litmus milk. Microscopic examination (Gram stain) after 4 days and again after 10 days at 37°C. did not show any spores although the organism from these media was found to survive a temperature of 92°C. for 20 minutes. There was a marked tendency to granular staining.

Examination of four cultures on lactose (Andrade) agar, incubated 3 days at 37°C. then stored for 2 weeks in the ice box also failed to disclose any spores when stained by Gram's method. As in the milk cultures there was a marked tendency to granular staining and the cells were resistant to heat, surviving 92° for 20 minutes.

We feel therefore that the organism does not form recognizable spores readily, if at all, on lactose media. This is of some practical significance in water work as it indicates the improbability of detecting spores of this bacillus and thereby differentiating it from B. coli by examination of stained mounts from confirmatory lactose agar plates.

TEMPERATURE RELATIONSHIPS

Growth is much more luxuriant on agar at 37°C. than at 20° to 22°C. At the lower temperature 4 days may be necessary before any growth is visible. No growth was visible after one week at 53°C.

CULTURAL CHARACTERS

Plain agar. On this substrate the character of growth varies with the age and consistency of the medium. On moist, fresh agar, there is a moderate, spreading, effuse, glistening, transparent, butyrous growth which is difficult to see if the medium is not perfectly clear. On dry (high agar content) or old agar the growth is filiform and almost opaque and in old cultures (2 weeks) it becomes membranous. The medium is not changed, there is no distinctive odor and no chromogenesis.

Lactose agar. On fresh lactose (Andrade) agar, the surface growth is almost invisible on account of its effuse character and transparency. It spreads rapidly over the surface forming acid, and acid and gas in butt.

Gelatin. At 37°C. gelatin was not liquefied in 48 hours.

At 20° to 22°C. gelative stabs showed a moderate growth which was best at the top and filiform along the line of puncture. Liquefaction was very slow, first becoming evident in from 14 to 20 days.

Tubes evenly inoculated on the surface and kept for 30 days (20° to 22°C.) showed but 2 mm. of liquefaction.

Broth. In nutrient broth at 37°C. there was but slight clouding and very little sediment. Surface growth was not evident until the third day when a pellicle was present. In sugar broth (glucose, lactose, sucrose, maltose and inulin) there was no surface growth even on long incubation. (14 days)

Potato. The reaction on potato was particularly striking. In 24–48 hours at body temperature, the entire mass of culture medium was covered with gas and in 4 to 7 days, the potato was almost completely digested. The diastatic action was very marked with all of the 14 cultures studied. The organism evidently produces a powerful pectinase, as the medium is entirely disintegrated.

COLONY CHARACTERISTICS

Plain agar. On nutrient agar surface colonies when well isolated, were irregular in form with a lobate edge. They may be described as amoeboid. The colonies were smooth, glossy and effuse, showed no distinct internal structure and quickly confluesced. It is generally difficult to discern the colonies due to the transparency of the growth.

Subsurface colonies resembled those of B. coli, i.e., they were circular or elliptical with an entire edge and showed a granular internal structure.

Litmus lactose agar. The surface colonies in 24 hours at 37°C. were faintly acid, otherwise resembling those described for plain agar.

The subsurface colonies were slightly acid resembling B. coli.

Incubation for 48 hours increased the acid reaction.

Endo agar. The medium employed was the product supplied by the Digestive Ferments Company. On this medium B. coli was observed to give a distinct red colony but only a slight metallic sheen, if any. Inoculation was made only on the surface.

The organism under consideration showed faint, but hardly discernible, growth in 24 hours at 37°C. After 48 hours small flat, round and amoeboid colonies about 1 mm. in diameter, pink to red, with a more intensely colored edge and center, were developed. We would certainly not consider it colon-like but these colonies would have to be fished in compliance with the United States Treasury Department Standard.

Eosin methylene blue agar. Two media were employed; the simplified E. M. B. of Levine and the Difco product. The results were similar. There was no growth in 24 hours and after 48 hours small pinhead, discrete colonies about ½ mm. in diameter with a distinct metallic sheen were present.

GROWTH IN LITMUS MILK

All cultures were inoculated into two different batches of litmus milk, made from skim milk powder, and incubated at 37°C.

The litmus was rapidly decolorized (24 hours) and the medium acidified. After 4 days, coagulation could be induced by heating. (Control tubes of B. coli also failed to coagulate unless heated). There was no further apparent change until 24 to 30 days incubation when evolution of gas followed by coagulation and extrusion of a clear whey was noticed in 10 of the cultures. It was thought that this reaction might be due to some contaminating organism. but microscopic examination of all of the tubes of one set of milk cultures showed only Gram negative long rods (and with a single exception, no spores) resembling the organism under consideration

Four cultures were inoculated into milk, covered with melted paraffine and heated at 80°C. for 10 minutes (the so-called sporogenes test). The litmus was reduced, there was no coagulation of the medium and no apparent change in 10 days at 37°C. At this time the milk coagulated on heating, and melting the paraffine seal by gently heating showed that there was but a very small amount of gas developed in the medium. The organism does not give the characteristic B. sporogenes or B. Welchii test.

INDOL FORMATION

Tests for indol were made on all strains in 7 days culture of broth and peptone with negative results.

NITRATE REDUCTION

All cultures reduced nitrates to nitrites when grown in nitrate broth for 5 days at 37°C.

FERMENTATION OF CARBOHYDRATES

Glucose neutral red broth. Four cultures and B. coli as a control were inoculated into (*a*) freshly heated neutral red glucose broth and (b) old unheated neutral red glucose broth, in Smith tubes, and incubated at body temperature.

In the freshly heated medium B. coli formed 25 per cent gas in 24 hours, but there was no reduction of the neutral red, while in the closed arm of the tubes of the old unheated medium the indicator was reduced to a canary yellow as is to be expected for B. coli.

The test cultures reacted in a similar manner, i.e. the dye was reduced in the unheated medium but not in the same medium which had been freshly heated. The gas formation was very meagre, in no instance being more than 5 per cent. There was no change in 48 hours.

Glucose peptone phosphate. Seven strains and B. coli, as a control, were tested for acid and gas production in 0.5 per cent peptone, glucose, dipotassium phosphate in Smith fermentation tubes.

The results are indicated in table 2.

Starch peptone water. Five strains were grown in 1 per cent arrowroot starch peptone water (Andrade indicator). There was very vigorous fermentation with acid and gas production. Both hydrogen and carbon dioxide were formed and the Voges Proskauer reaction was negative.

Lactose broth. Growth in lactose broth (with Andrade's indicator) always observed in Durham fermentation tubes was not as vigorous as in the glucose phosphate or starch mediums above. After 24 hours there was usually a distinct acidity in the open arm while the inner tube often showed but little acidity, (usually in lower end) and a small amount of gas. After 48 hours the entire medium becomes distinctly acid and 5 to 30 per cent gas may be obtained.

Inulin, sucrose and *maltose broths* were all fermented with acid and gas production. In all cases acid is first formed in open arm. Gas formation was particularly vigorous with inulin. It seems to be a peculiarity of this organism that it attacks the complex carbohydrates much more vigorously than the hexoses.

TABLE 2

Growth in Clark and Lubs medium (48 hours, 37°)

ORGANISM	(ANDRADE)	GAS PER CENT	CO_2	H_2 FLAME TEST	V. P.
14 F1	+	20	+	+	−
14 F2	+	5	+	+	−
14 F1	+	40	+	+	−
14 FA	+	35	+	+	−
14 BC	+	30	+	+	−
12 - 1	+	45	+	+	−
8	+	25	+	−	−
B. coli	+	45	+	+	−

FERMENTATION OF CARBOHYDRATES IN SOLID MEDIA

Nutrient agar containing 1 per cent of the test carbohydrate and Andrade's indicator were employed. Incubation was at 37°C. for 10 days. It was observed that the more complex test materials, starch, inulin, dextrin and glycogen were particularly vigorously fermented in 2 days (as indicated by the extent of disintegration of the agar) and that after 4 to 10 days, the acidity usually disappeared and the medium reverted to a distinct alkaline reaction. Dulcitol alone was not decomposed.

Carbohydrates were tested with the results indicated in table 3.

VOGES PROSKAUER AND METHYL RED REACTION

Tests for acetyl methyl carbinol and acidity to methyl red were made on all strains in Clark and Lubs medium after incubation at body temperature for 4 days. The Voges Proskauer reaction was negative in all cases. The reaction to methyl red was slightly alkaline or neutral. Growth was particularly vigorous in this medium and was accompanied by much foaming.

GROWTH IN SPECIAL MEDIA

Uric acid. There was no evidence of growth in Koser's uric acid medium after 4 days at 37°C.

TABLE 3

	14 UNKNOWN STRAINS			CONTROLS		(ACID AND GAS)
	Acid	Gas	Reversion	Para B.	B. coli	B. AEROGENES
Dextrose	+	+	Slow or negative	+	+	+
Levulose	+	+	Slow or negative	+	+	+
Galactose	+	++	Rapid, 4 day	+	+	+
Arabinose	+	+	Rapid, 4 day	+	+	
Mannose	+	+	Rapid, 4 day	+	+	
Xylose	+	++	Rapid, 4 day	+	+	+
Rhamnose	+	+	Rapid, 4 day	+	+	
Trehalose	+	+	Generally rapid	+	+	
Melezitose	+	+	Rapid, 4 day			
Lactose	+	+	4 to 10 day	−	+	+
Maltose	+	++	Rapid, 4 day	+	+	+
Sucrose	+	++	Rapid, 4 day	−	−	+
Mannitol	+	++	Rapid, 4 day	+	+	+
Glycerol	+	++	Rapid, 4 day	−	+	+
Dulcitol	−	−		+	−	−
Salicin	+	+	4 to 10 day	−	+	+
Dextrin	+	++	Rapid, 4 day	−	−	+
Inulin	+	++		−	−	−
Starch	+	++	Rapid, 4 day	−	−	+
Glycogen	+	++	Rapid, 4 day	−	−	−

Lactose bile. Six cultures were inoculated heavily into lactose bile (Difco). No gas was produced in 4 days.

Gentian violet lactose broth. In lactose broth containing gentian violet in a dilution of 1:100,000 there was no gas or other evidence of growth in 5 days at 37°C.

RESISTANCE TO HEAT

It has previously been mentioned that old cultures taken from lactose agar and milk survived a temperature of 92°C. for 20 minutes although no spores were visible on microscopic examination.

The following experiment was performed to check these observations: A loop of a 48 hour lactose (Andrade) broth culture was inoculated into each of 5 tubes of melted starch agar which were kept in a water bath at 89° to 91°C. After 10, 20, 30, 45 and 60 minutes, tubes were removed from the bath, cooled and incubated at 37°C. A Gram stain of the broth culture showed it to be a pure culture of long Gram negative rods and no spores were visible.

Plain and lactose agar culture suspended in broth were also heated as above. The results are given in table 4.

TABLE 4

Resistance of aerobic spore forming lactose fermenting bacilli to heat (89-91°C.)

TIME OF EXPOSURE	GROWTH IN STARCH AGAR (48 HOURS 37°C.)*		
	Inoculated from		
	Lactose agar	Lactose broth	Plain agar
minutes			
10	+	+	+
20	+	+	+
30	+	+	+
45	+	+	+
60	+	+	+
Microscopic examination of inoculum	Gram negative long rods. No spores	Gram negative long rods. No spores	Gram negative rods. Many spores

* Two cultures employed, 14B1 and 8.

IDENTITY OF ORGANISM

Meyer in 1919 described a sporing lactose fermenter which he isolated from Newport and Covington, Kentucky water. In many respects his organism is strikingly similar to the one here recorded (e.g., dulcitol is the only carbohydrate not attacked) and we thought that possibly they were the same, but rather important differences have been observed. The Meyer strain was non-motile, gave a positive Voges-Proskauer reaction, liquefied gelatin rapidly, and failed to reduce nitrates. The strain herein described is actively motile, negative for the Voges-Proskauer test, liquefies gelatine slowly and reduces nitrates very vigorously.

Two cultures have been described in connection with studies on acetone production which may be identical with the organism reported in this paper. The B. macerans of Schardinger was isolated in 1904 from potatoes. From the meagre description available, it cannot be differentiated from the strain under discussion. In 1919, Northrop, Ashe and Senior described an acetone producing organism, also isolated from potatoes, which they named B. acetoethylicum. It is said to differ from B. macerans in that the latter does not ferment galactose and levulose with NH_3 salts as a source of Nitrogen.

We have not been able to obtain cultures of *B. macerans* or B. acetoethylicum for comparison with our strains. In the published reports the fermentation of lactose (with gas) by *B. acetoethylicum* is not recorded while *B. macerans* is said to form gas in milk (presumably from lactose). We will therefore consider our strains tentatively as B. macerans.

SANITARY SIGNIFICANCE

Little is known as to the sanitary significance of B. macerans and closely related forms. Such organisms have been isolated from potatoes, retting flax, white flour and water. There is no record that they are present in the intestinal tract although a careful search may disclose them. Information as to the distribution of these sporing, lactose-fermenting forms capable of growing aerobically is now being gathered and the pathogenicity of the isolated strains is also being investigated. The organism isolated by Meyer was non-pathogenic.

In a chlorinated water B. macerans would be present long after the ordinary water-borne pathogens had been destroyed. The detection of B. macerans, in the absence of organisms of the colon group, in a treated water should therefore not be considered an indication of danger from such intestinal disturbances as typhoid fever or dysentery. The presence of these sporing organisms in water interferes seriously with the routine tests for B. coli with which they may be confused and is possibly responsible for the poor results sometimes reported in water purification.

SUMMARY

A Gram negative sporing bacillus capable of fermenting lactose and growing aerobically was isolated from two chlorinated surface water supplies in Iowa.

The morphological, cultural and physiological characteristics are detailed.

The strain resembles markedly that described by Meyer, differing with respect to rate of liquefaction of gelatin, nitrate reduction and the Voges-Proskauer test.

The organism should be of particular interest to water works operators because of its extreme resistance to chlorination, and because of the ease of confusion with the colon group in routine tests as ordinarily performed. Its presence in water may explain anomalous positive colon tests. Information as to its source is particularly desirable.

The cultures isolated are strikingly similar to *B. macerans* and *B. acetoethylicum*.

NOTE ON PATHOGENICITY

The *Bacillus acetoethylicum* of Northrup, Ashe and Senior was reported non-pathogenic to mice.

One of the similar organisms isolated in the course of this study was tested for pathogenicity on rabbits in the following manner:

An agar culture was scraped to remove the entire growth on its surface and the material suspended in physiological salt solution and added to the drinking water supplied to a rabbit. This process was repeated daily for a week. The test animal did not show any untoward symptoms.

Another rabbit was injected intravenously with one cubic centimeter of a live culture prepared by removing the growth of organisms from an agar slant as indicated above and suspending the material in 10 cc. of sterile salt solution. The animal developed no symptoms that would indicate bacterial infection.

We are therefore of the opinion that this organism is not a pathogenic form.

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